Appendix B

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Long-Lived Growth Hormone Receptor Knockout Mice: Interaction of Reduced Insulin-Like Growth Factor I/Insulin Signaling and Caloric Restriction

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Reduced IGF-I/insulin signaling and caloric restriction (CR) are known to extend the life span and delay age-related diseases. To address the interaction of these two interventions, we subjected normal (N) and long-lived GH receptor knockout (GHRKO) mice to CR for 20 months starting at weaning. We also used bovine GH transgenic (bGH Tg) mice, which overexpress GH and are short-lived and insulin resistant, for comparison. Circulating insulin and IGF-I levels were reduced by CR in N animals, whereas GHRKO animals exhibited very low insulin and undetectable IGF-I. Consistently, hepatic Akt phosphorylation was reduced by CR and was very low in GHRKO mice. bGH Tg mice exhibited increased active Akt. The forkhead box O1 (Foxo1) transcription factor was additively increased by CR and GHRKO at the mRNA level. However, Foxo1 protein levels were only elevated in GHRKO mice. The coactivator peroxisome proliferator-activated receptor-γ coactivator 1α was increased at both gene and protein levels in GHRKO mice. N-CR and GHRKO mice also exhibited increased phosphorylated cAMP response element-binding protein and active p38 compared with the N *ad libitum*-fed mice, and the levels of these proteins were greatly diminished in bGH Tg mice. The protein levels of the deacetylase sirtuin 1 (SIRT1) were elevated in the two CR groups and, unexpectedly, also in bGH Tg mice. These results suggest a major role for the Akt/Foxo1 pathway in the regulation of longevity in rodents. An activated gluconeogenic pathway and increased fat metabolism may be involved in mediating the effects of reduced somatotropic and insulin signaling on longevity. These results also add to the evidence that targeted disruption of the GH receptor/GH-binding protein gene and CR act via overlapping, but distinct, mechanisms. (*Endocrinology* 146: 851–860, 2005)

REDUCED IGF-I/INSULIN signaling is known to extend life span and delay age related diseases in different species ranging from worms to mammals. Studies in *Caenorhabditis elegans* provided the first evidence for the role of insulin-like signals in promoting longevity. Mutation in the gene *daf-2*, an insulin-like receptor, can prolong life in a manner that requires the activity of *daf*-16, a forkhead transcription factor. Insulin-like signals promote the phosphorylation and deactivation of DAF-16 (daver formation 16), which is a key regulator of heat and oxidative stress resistance, fat storage, fertility, and metabolism (reviewed in Refs. 1 and 2).

In mammals, data from GH-resistant, GH-deficient, and IGF-I receptor knockout mice provide evidence for the role of IGF-I in longevity. GH-impaired mice have very low circulating IGF-I and live longer than wild-type animals. Ames dwarf mice (Prop-1^{df}) and Snell dwarf mice (Pit-1^{dw}), both of which are GH, prolactin, and TSH deficient, live more than 40% longer than their wild-type counterparts. GH receptor/binding protein-deficient (GHRKO) mice also live much longer than wild-type animals. These GH-impaired mutant mice have very low serum IGF-I and reduced insulin and glucose levels (1). Female $Igf1r^{+/-}$ mice live 33% longer than wild-type females. $Igf1r^{+/-}$ mice are slightly smaller; have normal fertility, energy metabolism, food intake, and temperature; and resist oxidative stressors more efficiently than their wild-type littermates (1). Moreover, the fat-specific insulin receptor knockout mice have a 60% reduction in body fat and subsequent body weight reduction despite the normal food intake and live significantly longer than their nonmutated littermates (1).

Caloric restriction (CR) is the most robust and reproducible intervention that can extend life span and delay the onset of multiple age-related diseases (3), which makes it the gold standard in studying aging. Some of the phenotypes that are caused by mutations that reduce IGF-I/insulin signaling are also shared by animals subjected to CR, including reduced plasma insulin, IGF-I, and glucose; reduced fertility and body size; and delayed sexual maturation (2). In Ames dwarf mice, CR further extends the life span, suggesting differences in the mechanism by which these two interventions extend longevity (4). This is also supported by the findings that hemizygous rats overexpressing the antisense GH transgene have reduced function of the GH/IGF-I axis and extended life span, and that CR further extended the life of these long-lived transgenics (5).

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Abbreviations: AL, *Ad libitum* fed; AMPK, AMP-activated protein kinase; bGH Tg, bovine GH transgenic; CR, caloric restriction; CREB, cAMP response element-binding protein; Foxo1, forkhead box O1; GHR, GH receptor; GHRKO, GH receptor knockout; G6Pase, glucose-6-phosphatase; N, normal; p-, phosphorylated; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PPARγ, peroxisome proliferator-activated receptor-γ; SIRT1, sirtuin 1; SOD, superoxide dismutase; TBS, Tris-buffered saline; TBST, TBS plus 0.05% Tween 20.

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In this study we analyzed the interactions of reduced IGF-I/insulin signaling and long-term CR in the liver of long-lived GHRKO mice. The expression of molecules involved in hormonal regulation of glucose and lipid metabolism was studied. To contrast these findings with the impact of increased GH and IGF-I on the expression of the examined genes, we used bovine GH transgenic mice, which are short-lived and insulin resistant (reviewed in Ref. 6).

Materials and Methods

Antibodies

Antibodies for immunoblotting were obtained as follows: Akt2, phospho-Akt Ser^{473}, Foxo1, cAMP response element-binding protein (CREB), phospho-CREB Ser^{133}, AMP-activated protein kinase-α (AMPK-α), phospho-AMPK-α Thr^{172}, and p38 from Cell Signaling Technology (Beverly, MA); SIRT1 from Upstate Biotechnology, Inc. (Lake Placid, NY); active p38 from Promega Corp. (Madison, WI); peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC-1α) from Chemicon International (Temecula, CA); β-actin from Sigma-Aldrich Corp. (St. Louis, MO); and goat antirabbit and goat antimouse antibodies from Calbiochem (La Jolla, CA).

Animals

GHRKO mice and CR. GHRKO male mice and normal (N) littermate controls were produced in a closed colony derived from animals provided by Dr. J. Kopchick and maintained at Southern Illinois University by mating knockout (−/−) males with heterozygous (+/−) female carriers of the disrupted GHR/GH-binding protein gene. Animals were housed on a 12-h light, 12-h dark cycle at 22 ± 2 C and were fed (Lab Diet Formula 5008, Ralston Purina Corp., St. Louis, MO) and watered *ad libitum*, except as noted below. All animal procedures were approved by the laboratory animal care and use committee at Southern Illinois University School of Medicine. GHRKO mice and N siblings were fed either *ad libitum* (AL) or submitted to 30% CR protocol (eight animals per phenotype per diet) as described previously (7).

At 21 months of age, animals were anesthetized by isoflurane, bled by cardiac puncture, and decapitated. To avoid stressing animals, the time between taking the animal from the cage, anesthetizing, bleeding, and decapitation was minimized (~1 min). Livers were rapidly removed, quickly frozen on dry ice, and stored at −80 C until processed.

Bovine GH transgenic (bGH Tg) mice. Five-month-old male phosphoenolpyruvate carboxykinase (PEPCK)-bGH Tg mice and their N siblings, derived from animals provided by Dr. T. Wagner and J. Yun, were used in this study. Eight animals per genotype were killed as described above.

Plasma chemical analyses

Glucose concentrations were determined using a OneTouch Ultra glucose meter (LifeScan, Milpitas, CA). Insulin, leptin, and adiponectin were assessed by ELISA, and glucagon was determined using RIA kits from Linco Research, Inc. (St. Charles, MO). IGF-I and corticosterone were analyzed using ELISA kits from IDS, Inc. (Fountain Hills, AZ).

Total RNA extraction and cDNA transcription

Total RNA was extracted from the liver by the guanidinium thiocyanate-phenol-chloroform method (8). The RNA concentration was measured spectrophotometrically at 260 nm. One microgram of total RNA was electrophoresed on a 1.5% agarose gel to confirm RNA integrity. Potentially contaminating residual genomic DNA was eliminated using deoxyribonuclease I (Promega Corp.).

cDNA was made using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) as instructed by the manufacturer.

Real-time RT-PCR

Real-time RT-PCR amplification was carried out with the iQ SYBR Green PCR Super Mix (Bio-Rad Laboratories) using the SmartCycler (Cepheid, Sunnyvale, CA). The primers used are listed in Table 1. The real-time RT-PCR program included a 95 C denaturation step for 2 min, followed by 45 cycles of 95 C denaturation for 15 sec, 62 C annealing for 30 sec, and 72 C extension for 30 sec. Detection of fluorescent product was carried out at the end of the 72 C extension period. Melting curve and agarose gel electrophoresis were used to confirm PCR products. Data were analyzed and quantified using Cepheid SmartCycler software.

The reference gene used for normalization was β_2-microglobulin. Relative mRNA expression was calculated using the threshold cycle numbers (CT), *i.e.* $2^{-\Delta\Delta CT}$.

Protein extraction and immunoblotting

Total proteins were obtained from whole tissue homogenates. Approximately 100 mg liver samples were homogenized in 500 μl ice-cold homogenizing buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton 100, with protease inhibitors cocktail and phosphatase inhibitors cocktails (Sigma-Aldrich Corp.)] and spun at 16,000 rpm for 45 min. The supernatant was removed and stored at −80 C. Protein concentrations were determined using the bicinchoninic acid assay (Pierce Corp., Rockford, IL) according to the manufacturer's instructions.

For Western blotting, protein extracts were mixed with XT Sample Buffer (Bio-Rad Laboratories, Inc., Hercules, CA) and boiled for 5 min. Forty micrograms of total protein were separated electrophoretically according to size by SDS-PAGE using Criterion XT Precast Gel (26 wells; Bio-Rad Laboratories) for 60–80 min at 150 V. Subsequently, proteins were wet-transferred for 2 h at 100 V onto nitrocellulose membranes (Bio-Rad Laboratories) at 4 C. Membranes were rinsed briefly in Tris-buffered saline (TBS; pH 7.6) and blocked with 5% dry milk (or 3% BSA for phosphorylated proteins) in TBS plus 0.05% Tween 20 (TBST) for 1 h at room temperature. Blots were washed with TBST and incubated with the primary antibody diluted in the appropriate blocking solution at 4 C overnight with shaking. After incubation, blots were washed three times (15 min each) with TBST and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody. Horseradish peroxidase activity was detected using the ECL chemiluminescent reagent (Amersham Biosciences, Arlington Heights, IL). A minimum of six animals per group was analyzed, and Western blots were replicated at least twice. Photos of blots were taken with CCD camera (Hitachi Genetic Systems, Tokyo, Japan) and quantified for statistical analysis using GeneTools software (SynGene, Cambridge, UK).

TABLE 1. Primers used for gene expression analyses using real-time RT-PCR

Gene	GenBank accession no.	Forward (5′–3′)	Reverse (5′–3′)
Akt1	NM_009652	tacaaccaggaccacgacaa	tgatctccttggcatcctca
Akt2	NM_007434	gaggaccttccatgtagact	ctcagatgtggaagagtcac
Foxo1	NM_019739	cagatctacgagtggatggt	acttgctgtgaagggacaga
Pgc-1α	NM_008904	tacgcaggtcgaacgaaact	acttgctcttggtggaagca
Pepck	NM_011044	aaggagtacccattgagggt	catggctgctcctacaaaca
G6pase	NM_008061	tacagaagagcaagcccagg	acagttgcctaccagacaca
Gk	NM_010292	tgagtgcatctctgacttcc	ttggtccagttgagcaggat
Sod2	NM_013671	atgcagctgcaccacagcaa	ctcttcagcctgcactgaagt
B2m	NM_009735	aagtatactcacgccaccca	aagaccagtccttgctgaag

Immunoprecipitation-Western blot

For the analysis of phosphorylated AMPK, 1 mg of total protein was incubated with anti-AMPK antibody (Cell Signaling Technology) overnight at 4 C, and then 20 μl protein A agarose beads (50% bead slurry) were added to the mix and incubated for another 3 h at 4 C. The immunocomplexes were then spun for 30 sec, and the pellets were washed three times with TBST. Washed pellets were mixed with 10 μl XT Sample Buffer, and Western blotting was performed as previously described using anti-phospho-AMPK-α (Thr^{172}) antibody.

Statistical analysis

Data are expressed as the mean ± SE. The statistical evaluation was performed using two-factor ANOVA (phenotype and diet), followed by Fisher's protected least significant difference test as a *post hoc* test. A *t* test was also used to evaluate the effect of diet within phenotypes and phenotype within diet as well as analyzing results from bGH transgenic mice and their N siblings. $P < 0.05$ was considered significant. All statistical analyses were performed using StatView 5.0 software (SAS Institute, Inc., Cary, NC).

Results

Effects of GHRKO phenotype and CR on body growth

As expected, 30% CR resulted in consistent and significant reduction in the body weight of the animals. The difference in body weight between N-AL and N-CR mice reached its maximum around 7 months of age and remained stable until the 16 months of age, after which a slow decline started in the body weight of N-AL mice. In contrast, N-CR mice showed continuous increase in body weight until the termination of the study (Fig. 1). The reduction of body weight by CR was somewhat less pronounced between GHRKO mice (18%; $P < 0.05$) compared with that between normal mice (20%; $P < 0.001$) by the end of the study (Fig. 1 and Table 2).

Effects of GHRKO phenotype and CR on blood parameters

Glucose levels were reduced by CR in both phenotypes. KO-CR animals exhibited approximately 50% lower glucose than KO-AL ($P < 0.001$). There was no difference between the AL-fed groups ($P = 0.07$; Table 2).

Insulin was greatly reduced in N-CR, KO-AL, and KO-CR (66%, 79%, and 90%, respectively) compared with N-AL. CR also resulted in significant reduction of insulin levels in GHRKO animals compared with their AL siblings ($P < 0.01$).

As expected from previous studies (9), CR also resulted in a significant reduction (38.4%) in plasma IGF-I levels in normal animals, whereas IGF-I levels in GHRKO mice were undetectable regardless of their diet (Table 2).

Glucagon levels were numerically, but not significantly, elevated in GHRKO compared with N animals (Table 2).

Analysis of the stress hormone, corticosterone, under conditions of mild stress showed elevated levels in GHRKO mice ($P < 0.001$; Table 2).

Circulating leptin levels were reduced by CR in both N and GHRKO mice ($P < 0.01$). KO-AL animals exhibited higher leptin levels compared with N-AL mice ($P < 0.05$; Table 2).

Adiponectin, a plasma protein secreted specifically from adipocytes, was elevated in the plasma of GHRKO mice ($P < 0.001$) with no diet effects (Table 2).

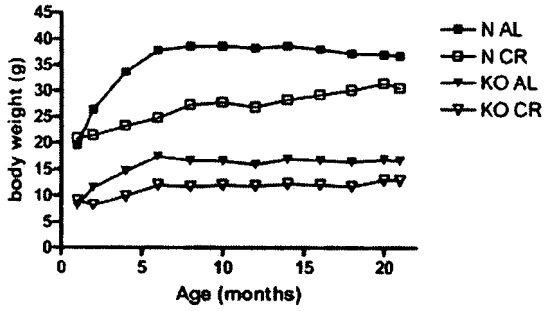

FIG. 1. Growth curves of GHRKO (KO) and N mice fed either *ad libitum* (N-AL and KO-AL) or subjected to CR (N-CR and KO-CR) for 20 months. Each *time point* represents the average weight of eight animals per group.

Total and phosphorylated AMPK protein levels are elevated in GHRKO mice

The total AMPK protein level was significantly higher in GHRKO mice ($P < 0.001$) with no diet effect in either phenotype (Fig. 2). The phosphorylated form of AMPK (Thr^{172}) was also significantly elevated in GHRKO animals ($P < 0.001$), with no diet effect (Fig. 2).

Akt/PKB activation is reduced by CR and GHRKO and increased in bGH Tg mice

The mRNA expression of both Akt1 and Akt2 did not appear to be altered by diet; however, KO animals showed elevated Akt mRNA (Fig. 3, A and B). Total Akt1 and Akt2 protein levels were not altered in all groups. However, phosphorylated Akt at Ser^{473} was significantly reduced by diet ($P < 0.05$) and phenotype ($P < 0.0001$; Fig. 3C). The phosphorylation of Akt Ser^{473} was reduced in N-CR ($P < 0.05$) and to a much greater extent in GHRKO animals ($P < 0.0001$) compared with N-AL controls. In N-AL animals there was slight variation in the amount of phosphorylated Akt, possibly due to the fed state in which the animals were killed. In GH Tg mice, Akt phosphorylation was significantly increased compared with that in their N-AL counterparts ($P < 0.001$; Fig. 3D).

GHRKO, but not CR, increases Foxo1 expression

The hepatic expression of Foxo1 in our study showed stimulating effects of CR ($P < 0.01$) and GHRKO phenotype ($P < 0.001$) on Foxo1 mRNA, whereas total Foxo1 protein levels were elevated in GHRKO mice ($P < 0.001$) with no diet effects (Fig. 4, A and B). Foxo1 total protein in bGH Tg mice did not differ from that in normal mice (Fig. 4C).

Increased gluconeogenic enzymes and superoxide dismutase-2 (SOD2) mRNA expression in GHRKO mice

We also analyzed some of the genes that are activated by Foxo1. Two-way ANOVA of phosphoenolpyruvate carboxykinase (PEPCK) mRNA revealed diet and phenotype effects with the levels in CR and GHRKO mice being higher ($P < 0.001$). KO-AL exhibited increased *pepck* expression compared with N-AL ($P < 0.0001$), and CR further increased it in KO-CR ($P < 0.0001$, KO-AL *vs.* KO-CR; Fig. 5A).

Glucose-6-phosphatase (G6Pase) gene expression was increased in GHRKO mice ($P < 0.0001$), but was not affected

TABLE 2. Body weight and various plasma parameters from normal and GHRKO mice subjected to caloric restriction

	N-AL	N-CR	KO-AL	KO-CR
Body weight (g)	36.7 ± 1.6^a	29.5 ± 0.5^b	15.8 ± 1.1^c	13 ± 0.3^c
Glucose (mg/dl)	189.4 ± 9.6^a	139.7 ± 14.1^b	$155 \pm 14.1^{a,b}$	74.8 ± 11.5^c
Insulin (ng/ml)	4.2 ± 0.7^a	1.4 ± 0.2^b	0.9 ± 0.1^b	0.4 ± 0.1^c
IGF-I (ng/ml)	315 ± 29^a	194 ± 33^b	ND	ND
Corticosterone (ng/ml)	29.0 ± 8.1^a	44.3 ± 11.2^a	83.6 ± 18.6^b	86.3 ± 11.6^b
Glucagon (ng/ml)	62.8 ± 8.4	66.4 ± 11.6	75.8 ± 5.6	78.5 ± 9.8
Leptin (ng/ml)	5.9 ± 1.0^a	2.3 ± 0.4^b	9.9 ± 1.8^c	$4.7 \pm 0.6^{a,b}$
Adiponectin (μg/ml)	5.5 ± 0.4^a	5.1 ± 0.3^a	7.7 ± 0.3^b	8.6 ± 0.8^b

Different superscripts denote significant difference at $P < 0.05$. Data represent the mean ± SE.

by CR (Fig. 5B). Similarly, the mitochondrial antioxidant enzyme MnSOD (SOD2) was only increased in GHRKO animals ($P < 0.001$; Fig. 5C).

PGC-1α expression is augmented by CR and GHRKO and is diminished in bGH Tg mice

To gain additional insight about other proteins that are involved in the regulation of gluconeogenesis, we assessed the gene expression of PGC-1α in the animals under study. PGC-1α gene expression was elevated in GHRKO animals ($P < 0.001$; Fig. 6A). At the protein level, however, PGC-1α was additively elevated by both CR and phenotype ($P \leq 0.013$, N-AL *vs.* N-CR; Fig. 6B). In contrast, bGH Tg mice exhibited reduced PGC-1α protein levels compared with normal animals ($P < 0.01$; Fig. 6C).

Active p38 MAPK is increased in CR and GHRKO, but reduced in bGH Tg, mice

We also assessed the hepatic level of active p38. Phospho-p38 MAPK (Thr^{180}/Tyr^{182}) was elevated in N-CR ($P < 0.02$) and GHRKO ($P < 0.001$) animals compared with N-AL mice (Fig. 7A). In contrast, bGH Tg mice exhibited diminished active p38 ($P < 0.001$; Fig. 7B).

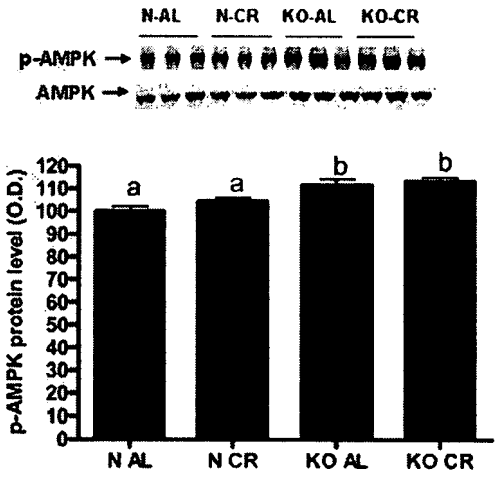

FIG. 2. Total and phosphorylated AMPK protein levels in GHRKO (KO) and N mice fed *ad libitum* (AL) or subjected to CR, as measured by immunoprecipitation and Western blotting. In this and subsequent figures, data are expressed relative to the mean value for the N-AL group. Group values are the mean ± SE. Groups that do not share a superscript are significantly different ($P < 0.05$). At least six animals per group were analyzed. Results of statistical analyses are given in the text. Representative blots are shown.

CREB phosphorylation is increased in CR and GHRKO, but diminished in bGH Tg, mice

Two-way ANOVA of the phosphorylation of cAMP response element-binding protein (p-CREB) showed elevation by CR ($P < 0.001$) and GHRKO ($P < 0.001$; Fig. 8A). N-CR showed increased p-CREB ($P < 0.01$) compared with N-AL mice. KO-AL mice also exhibited increased p-CREB compared with N-AL ($P < 0.01$), and CR further augmented p-CREB in GHRKO animals ($P < 0.05$; Fig. 8A). In bGH Tg animals, p-CREB was greatly reduced compared with that in N siblings ($P < 0.001$; Fig. 8B).

SIRT1 is only induced by CR

We also analyzed the protein expression of SIRT1. CR, but not GHRKO, resulted in increased SIRT1 protein levels ($P < 0.001$). SIRT1 was increased in N-CR and KO-CR compared with the corresponding AL groups (Fig. 9A). Assessment of SIRT1 in bGH Tg mice revealed that bGH Tg mice have elevated SIRT1 protein levels ($P < 0.01$; Fig. 9B).

Discussion

In this study we explored the overlapping as well as divergent effects of a mutation that reduces IGF-I and insulin levels and long-term CR. The animals used in this study have a disruption in GHR and GH-binding protein; hence, these animals are GH resistant and, as a consequence, have dramatically reduced IGF-I levels in the circulation. These animals are long-living, have reduced glucose and insulin levels, and have increased insulin sensitivity (10–12). To further elucidate the impact of increased GH and IGF-I on the parameters under investigation, we also used mice that overexpress bGH. bGH Tg mice have increased or normal glucose levels, have increased circulating IGF-I and insulin levels, are insulin resistant, and have a shortened life span (6).

In the present study CR resulted in decreased glucose levels in both normal and GHRKO animals. There was no difference between the AL-fed groups, possibly due to the fact that the animals in this study were not fasted before death to avoid comparing the effects of acute fasting and its associated stress with chronic CR.

CR is reported to increase the sensitivity to insulin (3). Insulin sensitivity is also increased in GHRKO mice (12). However, glucose tolerance in GHRKO mice is reduced, implying a reduced insulin reserve and/or reduced insulin secretion in response to glucose (12). Reduced glucose-induced insulin secretion in GHRKO animals could be attributed to the reduced circulating levels of insulin and IGF-I.

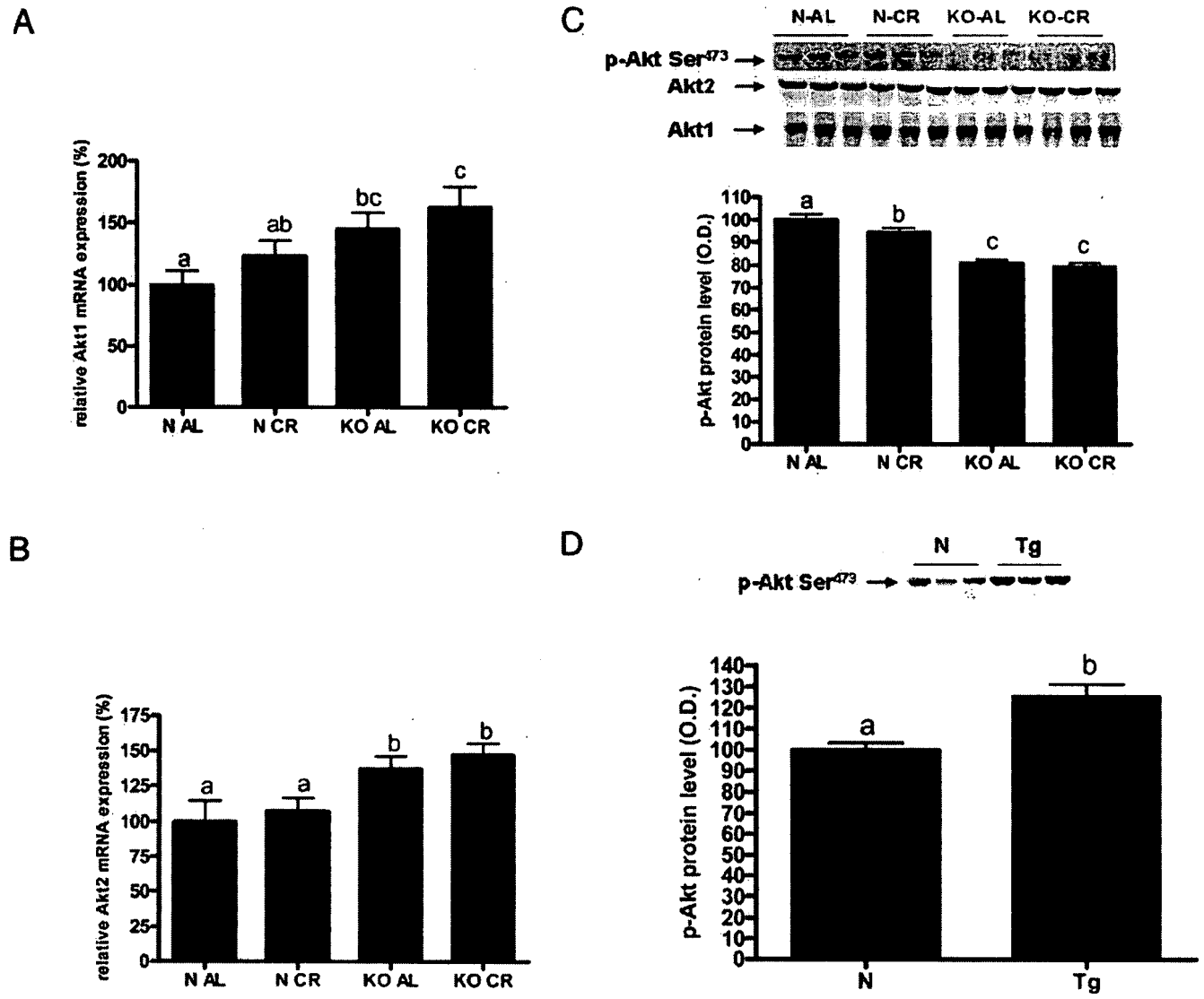

FIG. 3. Akt expression in GHRKO (KO) and N mice subjected to CR. Relative Akt1 (A) and Akt2 (B) mRNA expression as measured by real-time RT-PCR. C, Akt protein levels as measured by immunoblotting. Total and phosphorylated Akt Ser^{473} in normal and GHRKO mice. D, Akt Ser^{473} phosphorylation in N and GH Tg mice.

It was recently reported that loss of either insulin receptor or IGF-I receptor caused a total loss of glucose-mediated insulin secretion but not that mediated by depolarizing agents in cultured β-cells (13). Moreover, it was recently reported that GHRKO mice have reduced pancreatic β-cell mass per body weight, whereas α-cell mass is increased compared with that in wild-type animals (12), which might explain the reduced insulin and normal or increased glucagon levels. It is very well established that a chronic excess of GH has antiinsulin effects. Mice with liver-specific IGF-I gene knockout have reduced peripheral IGF-I levels and increased circulating GH and are insulin resistant, which can be alleviated by decreasing GH signaling (14). It is expected, therefore, that impaired GH action in GHRKO mice is responsible for increased insulin sensitivity and reduced glucose-stimulated insulin secretion.

The increased circulating corticosterone levels in GHRKO mice in this study make it tempting to speculate that reduced GH/IGF-I signaling would increase the animal's ability to cope with stress. In support of this possibility, fibroblasts from hypopituitary dwarf mice exhibit increased resistance to multiple forms of cytotoxic stress (15).

In the liver, insulin controls hepatic glucose production, thereby preventing unnecessary elevations in fasting plasma glucose levels. The gluconeogenesis pathway, which is activated during fasting and chronic CR, also appeared to be activated in GHRKO mice. The decreased levels of insulin and the concomitant elevated corticosterone and perhaps also glucagon levels would be expected to promote gluconeogenesis.

KO-AL animals also exhibited increased circulating leptin levels and CR reduced it in both phenotypes (Table 2). This could be attributed to the increased adiposity in male GHRKO. It was shown that male GHRKO have increased fat body composition compared with normal males (34.6% *vs.*

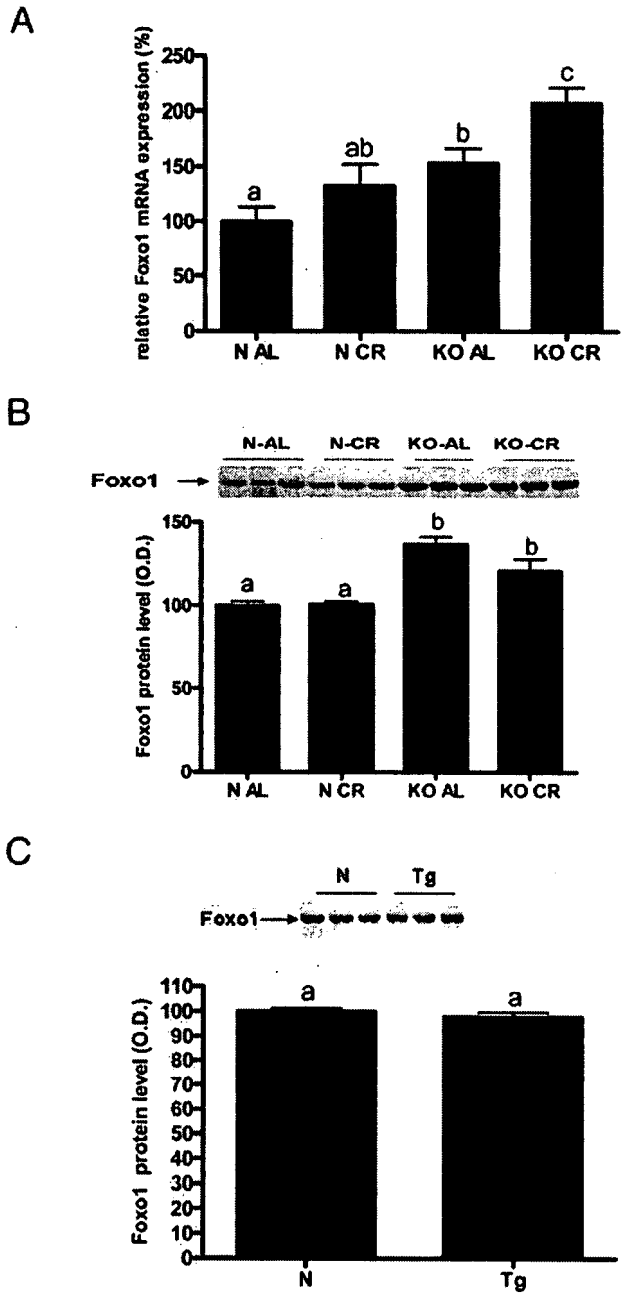

FIG. 4. Foxo1 expression in GHRKO (KO) and N mice subjected to CR. A, Relative Foxo1 mRNA expression as measured by real-time RT-PCR. B, Total Foxo1 protein levels as quantified by immunoblotting. C, Total Foxo1 protein levels in N and GH Tg mice.

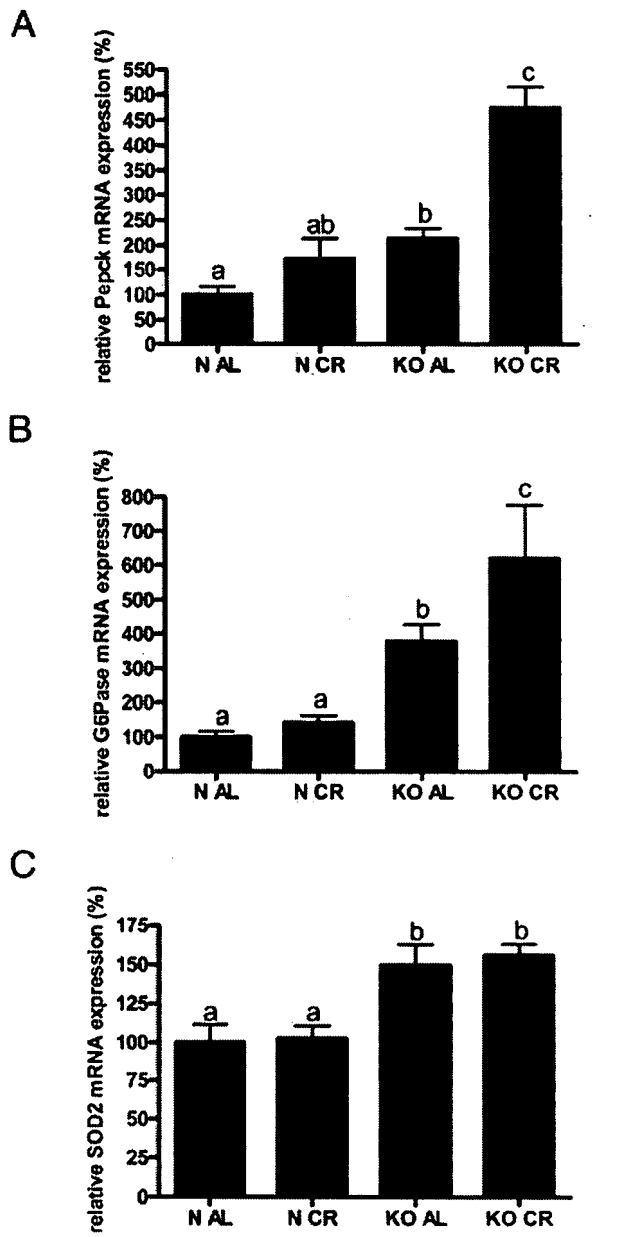

FIG. 5. The mRNA expression of PEPCK (A), G6Pase (B), and SOD2 (C) as measured by real-time RT-PCR in N and GHRKO (KO) mice subjected to CR.

18.8%, respectively) (16). It was shown in rats that the administration of human IGF-I decreased leptin mRNA in epididymal fat pads (17). Moreover, GH administration to GH-deficient patients resulted in decreased leptin levels (18). Higher levels of leptin in GHRKO animals could be attributed to increased adiposity and decreased IGF-I levels, whereas CR, by decreasing fat mass, is expected to reduce leptin levels.

Adiponectin, a plasma protein secreted specifically from adipocytes, is elevated in the plasma of KO mice with no diet effects. This adipokine is implicated in the regulation of lipid metabolism and glucose homeostasis in liver, skeletal muscle, and adipocytes and is thought to play role in improving insulin sensitivity (19). Fasting was recently found to increase the expression of adiponectin receptors in liver and skeletal muscle of mice (20), and insulin appeared to negatively regulate adiponectin receptor expression in hepatocytes and myocytes *ex vivo* in a phosphatidylinositol 3-kinase/Foxo1-dependent pathway (20). Adiponectin is implicated in the insulin-sensitizing effects of thiazolidinediones, specific synthetic ligand activators of peroxisomal proliferator-activated receptor-γ that improve glucose tolerance and insulin sensitivity (21, 22). Moreover, adiponectin elicits its action through AMPK (23), a mechanism

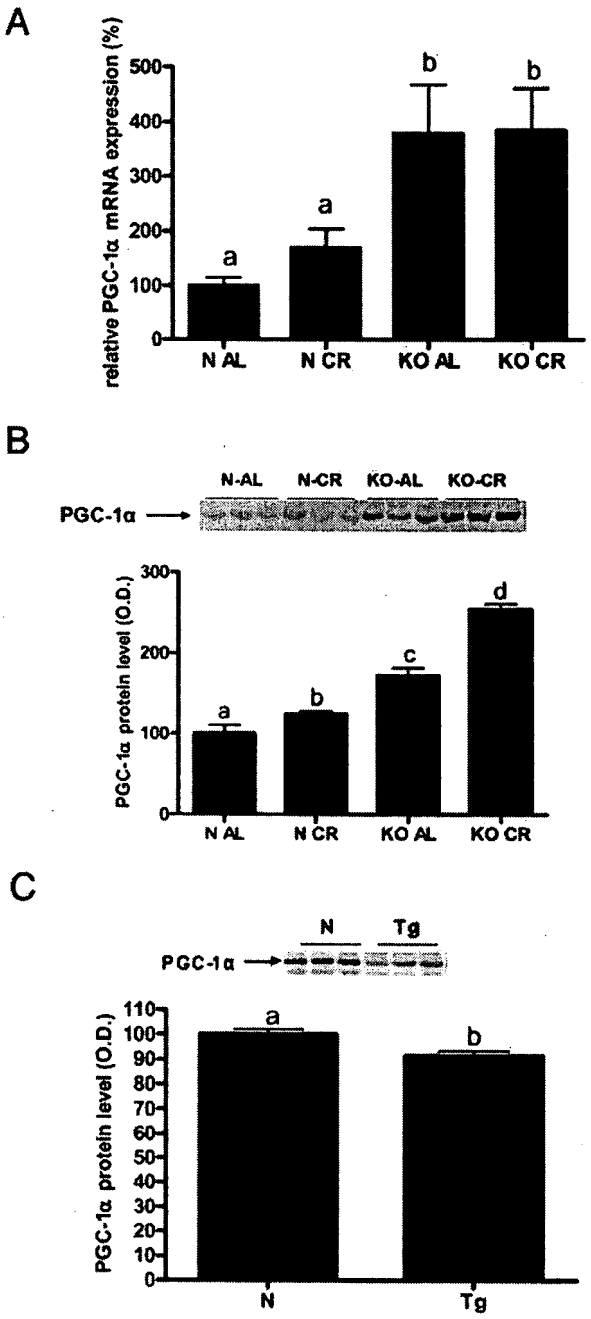

FIG. 6. PGC-1α expression in GHRKO (KO) and N mice subjected to CR. A, RT-PCR analysis of PGC-1α mRNA. Hepatic PGC-1α protein amount in N *vs.* KO mice (B) and N *vs.* GH Tg mice (C).

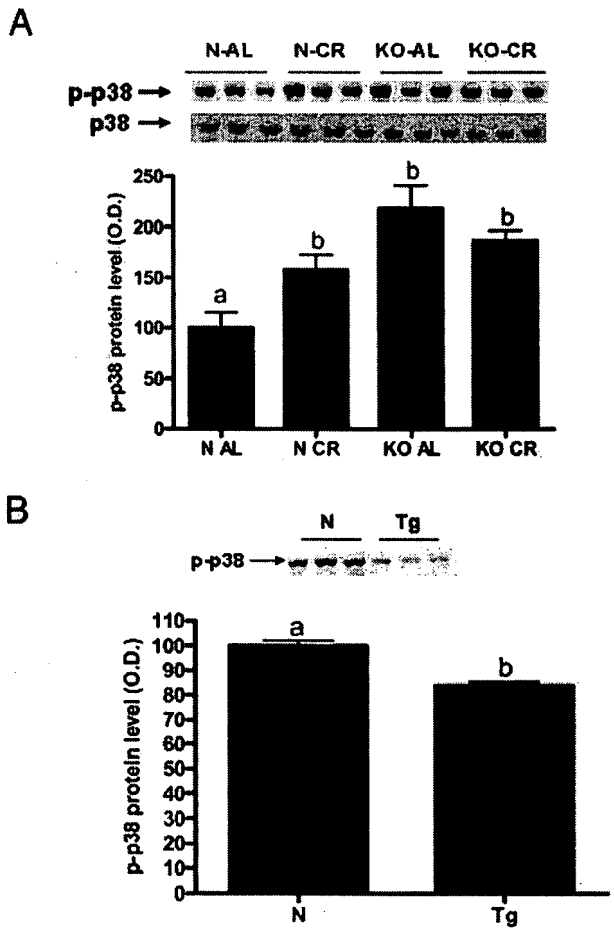

FIG. 7. Total and phosphorylated p38 protein levels in GHRKO (KO) and normal (N) mice subjected to CR (A) and phosphorylated p38 in GH Tg and N mice (B).

by which metformin is thought to induce its antidiabetic effects (24–26). Collectively, adiponectin has insulin-like actions in terms of increased glucose uptake and lipid metabolism, which could compensate for the reduced insulin in GHRKO mice.

Analysis of AMPK protein showed elevated levels of total and phosphorylated protein in GHRKO mice. AMPK is activated when the ATP:ADP ratio is reduced by cellular stresses that hinder ATP production or increase ATP consumption (27). AMPK mediates the actions of leptin and adiponectin in promoting fat oxidation, which prevents fat accumulation in tissues other than adipose tissue (27).

In *C. elegans*, genetic analyses have demonstrated that reduction of function mutations in Akt1 and Akt2 extend the longevity of the nematode in a Daf-16-dependent manner (28). Moreover, Akt was recently reported to negatively regulate the lifespan of primary cultured human endothelial cells in a p53/p21-dependent pathway, and this action was mediated at least in part by FOXO3a, which regulates cellular reactive oxygen species levels (29). Reduced Akt activity in hepatocytes from N-CR and GHRKO mice is likely to be beneficial in protection against cellular stresses by activating Foxo proteins. In contrast, Akt phosphorylation in bGH Tg mice was increased in parallel to elevated insulin and IGF-I levels. Reduced active Akt in GHRKO mice and increased active Akt in short-lived, insulin-resistant bGH Tg mice support the idea that Akt is a major player in cellular senescence and overall organismal aging (30). Perhaps Akt in bGH Tg mice is still capable of activating senescence mechanisms while the metabolic effects of insulin are repressed.

The forkhead transcription factor Daf-16, which is phosphorylated and thereby inactivated by Akt, plays an essential role in the exceptional longevity of *C. elegans* by regulating heat and oxidative stress resistance, fat storage, develop-

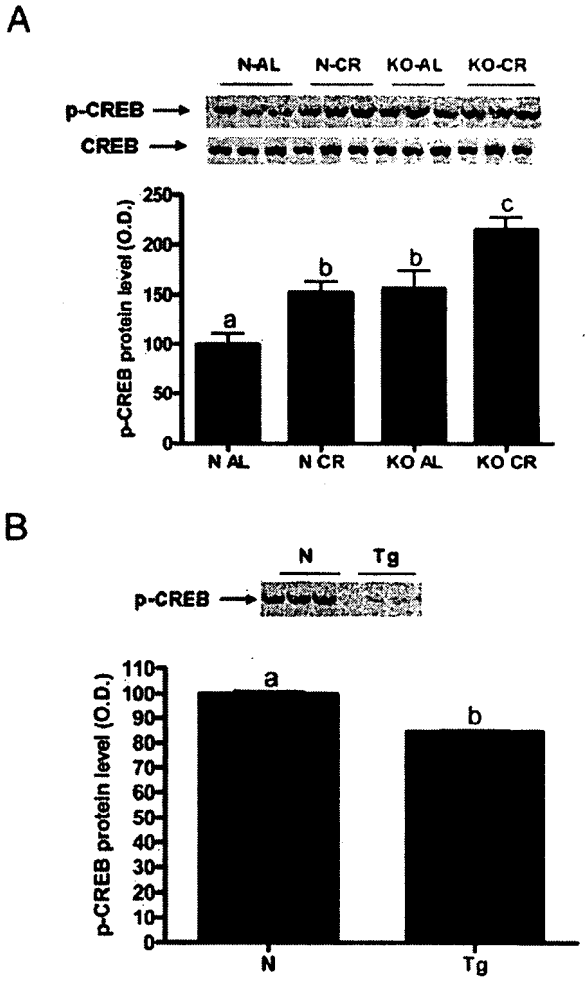

FIG. 8. Levels of total and phospho-CREB in GHRKO (KO) and N mice subjected to CR (A) and in GH Tg mice (B).

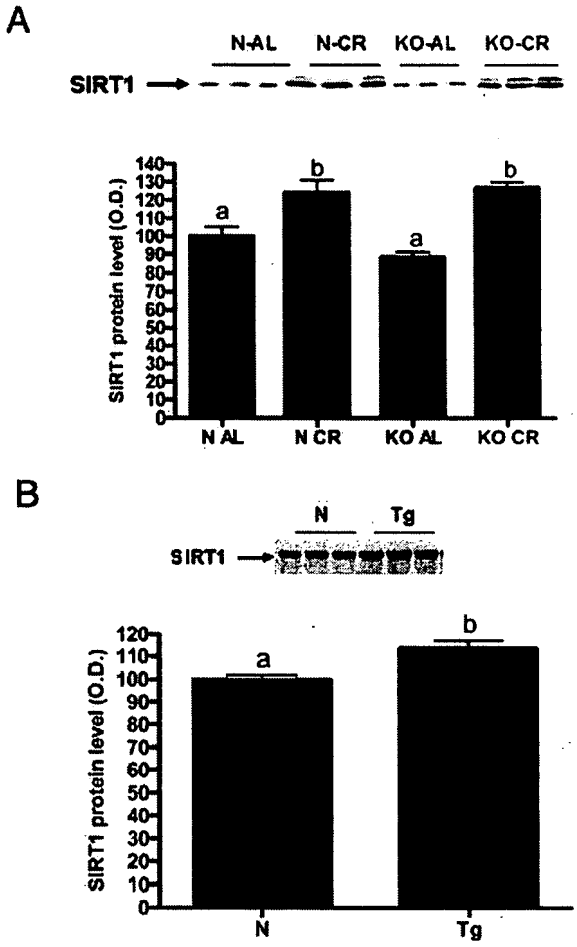

FIG. 9. SIRT1 protein expression in GHRKO (KO) and N mice subjected to CR (A) and in GH Tg mice (B).

mental arrest, fertility, and metabolism (reviewed in Ref. 1). GHRKO mice exhibited elevated Foxo1 protein levels, whereas bGH Tg mice showed no difference. Foxo1 regulation by GHRKO, but not by CR, along with decreased active Akt suggests more activity of this transcription factor in cellular protection and other cellular functions. CR did not affect Foxo1 protein levels in normal animals; however, the amount of active Foxo1 protein is expected to be higher due to reduced Akt activity. In contrast, the amount of active Foxo1 in bGH Tg mice is likely to be reduced.

Among Foxo1 target genes are the gluconeogenic genes PEPCK and G6Pase (reviewed in Ref. 31). Foxo1 appears to be one of the mechanisms through which insulin and its opponents, glucocorticoids and glucagon, regulate gluconeogenesis (31). Inhibition of hepatic Foxo1 activity by adenovirus-mediated gene transfer of a dominant negative mutant Foxo1 is associated with reduced hepatic gluconeogenesis and improved fasting glycemia in diabetic rodents (31). Foxo1 was also found to physically and functionally bind to PGC-1α, and this complex activates PEPCK and G6Pase, a process that is regulated by insulin (31). Taken together, increased Foxo1 activity in GHRKO and possibly N-CR would suggest increased hepatic glucose production and altered lipid metabolism.

Analyses of PEPCK and G6Pase mRNA expression in our study showed that their expression was increased in GHRKO mice, and CR further increased it only in these animals, possibly due to much reduced glucose levels in KO-CR mice. Additive effects of CR and reduced IGF-I/insulin were also reported from Ames dwarf mice in terms of genes responsible for gluconeogenesis, protein turnover, and lipid β-oxidation (32). Increased gluconeogenesis might explain the impaired tolerance of glucose challenge in GHRKO animals.

Increased oxidative stress resistance is proposed as a mechanism by which CR extends longevity (3). It is likely that this CR effect is also shared by mutations that reduce IGF-I/insulin signaling. GHRKO mice exhibit increased hepatic catalase and Cu/Zn-SOD1 activities (33). In the present study, MnSOD (SOD2) mRNA expression was increased in GHRKO mice, which could reflect activation of transcription by Foxo transcription factors (34). This also supports the idea that GHRKO mice have more protection against cellular stresses at least partially via increased Foxo protein activity.

PGC-1α regulates a wide range of processes involved in energy production and utilization, including the gluconeogenic pathway and β-oxidation of fatty acids. PGC-1α was

found to be highly elevated in liver-specific insulin receptor KO mice, streptozotocin-injected mice, and *ob/ob* mice (which are insulin resistant), indicating induction of PGC-1α expression by reduced insulin signaling (reviewed in Ref. 35). PGC-1α was reported to be strongly induced by fasting in mouse liver and in response to glucocorticoids and cAMP in primary liver culture (36). High PGC-1α levels in GHRKO animals might indicate increased gluconeogenesis, fatty acid oxidation, and mitochondrial biogenesis and respiration.

PGC-1α contains a negative regulatory region that reduces its transcriptional activity, which can be alleviated by the phosphorylation of this regulatory region by the stress-activated MAPK, p38. This phosphorylation increases its stability and transcriptional activity (reviewed in Ref. 35). N-CR and GHRKO animals have elevated active p38, whereas bGH Tg mice have severely reduced active p38, suggesting overlapping mechanisms of action of reduced IGF-I/insulin signaling and CR. Besides its role in activating PGC-1α, p38 has numerous other functions related, but not limited, to cellular stress responses.

The inappropriate elevation of Foxo1 and PGC-1α levels in diabetic models seems to be secondary to insulin resistance and the lack of insulin-induced suppression of these transcription factors in response to elevated glucose, which further exacerbates the situation. In GHRKO animals, however, increased expression of these factors presumably reflects a physiological need for hepatic glucose production, as indicated by reduced or normal plasma glucose levels, and could secondarily help in providing cellular protection.

Activation of the cAMP signaling pathway by glucagon and other factors leads to the phosphorylation of CREB, which recruits the coactivators CREB-binding protein and p300, thus activating transcription (reviewed in Ref. 37). CREB was shown to activate gluconeogenic and fatty acid oxidation programs during fasting by stimulating the expression of PGC-1α in response to glucagon and glucocorticoids (38). Recently, insulin was reported to phosphorylate CREB-binding protein, preventing it from being recruited to CREB, thus inhibiting CREB transcriptional activity (39). Moreover, CREB was also found to inhibit hepatic PPARγ expression and lipogenesis during fasting (40). CR and GHRKO phenotype resulted in increased CREB phosphorylation. In contrast, CREB phosphorylation was severely reduced in bGH Tg mice. These findings along with elevated Foxo1 and PGC-1α levels suggest a parallel metabolic shift in glucose and lipid metabolism.

It is well documented that aging is associated with decreased protein turnover and renewal and that CR restores this decline in protein turnover and enhances protein renewal (41). Besides being a defense mechanism against damaged and toxic proteins, protein turnover can be used as a source of *de novo* glucose synthesis.

Studies in the budding yeast *Saccharomyces cerevisiae* revealed the importance of Sir2 in the life-extending effects of CR. Sir2 is an NAD-dependent histone deacetylase that is required for chromatin silencing and extended longevity of yeast (41). Sir2 activity has also been implicated in *C. elegans* longevity; the gain of function Sir2 mutation extended the worm's life span in a Daf-16-dependent manner (42). These studies revealed the convergence of the effects of reduced IGF-I/insulin and CR. Recent studies showed that Foxo proteins are deacetylated and thus regulated by SIRT1, one of the mammalian Sir2 orthologs (43–45). These studies revealed that deacetylation of Foxo1 by SIRT1 has differential effects on Foxo1 target genes. SIRT1 was reported to be induced by CR in rats (46). This response to CR was attenuated by insulin and IGF-I in cultured cells (46). We found that CR also induces SIRT1 protein expression in mouse liver. It was expected in GHRKO animals that SIRT1 protein levels would be elevated because IGF-I and insulin levels are reduced; however, there was no difference in SIRT1 protein levels in the AL groups, indicating divergent effects of CR and GHRKO mutation. SIRT1 was recently reported to attenuate adipogenesis and activate fat mobilization in white adipocytes by repressing PPARγ (47). Moreover, *Sirt1*$^{+/-}$ mice manifested blunted fat mobilization (47). Apparently, Foxo1, cAMP, and SIRT1 pathways converge on PPARγ nuclear receptor, increasing fat mobilization and oxidation. Surprisingly, SIRT1 protein levels were elevated in bGH Tg mice. It is interesting to speculate that increased expression of SIRT1 in bGH Tg mice may mediate or augment the effects of supraphysiological GH levels on lipolysis and body composition.

In summary, GH resistance and CR have independent as well as overlapping effects. The findings of the current work can be summarized as follows. 1) Reduced active Akt in N-CR and GHRKO mice and increased active Akt in bGH Tg mice support the idea that Akt plays a major role in aging. 2) Many of the molecules involved in the activation of gluconeogenesis were up-regulated in GHRKO mice, including Foxo1, PGC-1α, CREB, PEPCK, and G6Pase, suggesting increased hepatic glucose production and fatty acid oxidation. 3) The deacetylase SIRT1 is increased in CR, but not in GHRKO, animals. 4) Increased Foxo1 proteins and MnSOD gene expression suggest increased protection in the liver of GHRKO mice against oxidative stress, which may play a role in the extended longevity of these mice.

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